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Transforming Growth Factor β1 Production by CD4$^+$ CD25$^+$ Regulatory T Cells in Peripheral Blood Mononuclear Cells from Healthy Subjects Stimulated with *Leishmania guyanensis*

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Transforming growth factor β (TGF-β) has been shown to be a central immunomodulator used by leishmaniae to escape effective mechanisms of protection in human and murine infections with these parasites. However, all the information is derived from studies of established infection, while little is known about TGF-β production in response to *Leishmania* stimulation in healthy subjects. In this study, TGF-β1 production was demonstrated in peripheral blood mononuclear cells from healthy subjects never exposed to leishmaniae in response to live *Leishmania guyanensis*, and the TGF-β1-producing cells were described as a distinct subpopulation of CD4$^+$ CD25$^+$ regulatory T cells. The suppressive properties of CD4$^+$ CD25$^+$ T cells were demonstrated in vitro by their inhibition of production of interleukin 2 (IL-2) and IL-10 by CD4$^+$ CD25$^-$ T cells in the presence of either anti-CD3 or *L. guyanensis*. Although neutralization of TGF-β1 did not reverse the suppressive activity of CD4$^+$ CD25$^+$ T cells activated by anti-CD3, it reversed the suppressive activity of CD4$^+$ CD25$^+$ T cells activated by *L. guyanensis*. Altogether our data demonstrated that TGF-β1 is involved in the suppressive activity of *L. guyanensis*-stimulated CD4$^+$ CD25$^+$ T cells from healthy controls.

Human infections with *Leishmania* species induce diseases ranging from cutaneous leishmaniasis to mucosal and visceral leishmaniasis, and the clinical outcomes depend largely on the specific immune responses to *Leishmania* antigens and particularly on cytokine production. In the murine model of infection with *Leishmania major*, the involvement of the development of CD4$^+$ Th1 cells producing interleukin 2 (IL-2) and gamma interferon (IFN-γ) and of CD4$^+$ Th2 cells producing IL-4, IL-10, and IL-13 in resistance and susceptibility, respectively, has been clearly demonstrated (28). However, in humans, the roles of Th1 and Th2 CD4$^+$ T cells and the cytokines they produce are not yet well understood. IL-10 production has been demonstrated in human cutaneous leishmaniasis due to *L. braziliensis*, and its down-regulation of IFN-γ production has been proposed to explain disease (29). Transforming growth factor β (TGF-β) is also a well-characterized antiinflammatory cytokine with antiproliferative and antigen-presenting cell (APC)-deactivating properties which has been implicated in human leishmaniasis (5). Recent data have demonstrated, however, that in vitro TGF-β could poorly inhibit IFN-γ production by peripheral blood mononuclear cells (PBMC) from leishmaniasis patients, suggesting that exogenous TGF-β was not able to reverse IFN-γ antiparasite effector mechanisms (2). Therefore, the exact role of TGF-β in disease susceptibility is still unclear.

Both IL-10 and TGF-β are secreted by mouse and human regulatory T cells (T_{reg} cells) (30), which represent 5 to 10% of the peripheral CD4$^+$ T cells. The suppressive activity of CD4$^+$ CD25$^+$ T_{reg} cells has been reported both in vitro and in vivo. Suppression was shown to depend on cell contact in vitro, while the cytokines IL-10 and TGF-β appeared critical in their suppressive activity in vivo. CD4$^+$ CD25$^+$ T_{reg} cells are restricted by self-antigens, but their role in the control of immune responses against several pathogens has been described recently (26). An important role for CD4$^+$ CD25$^+$ T_{reg} cells has been reported in the control of *L. major*-specific immune responses in both susceptible BALB/c and resistant C57BL/6 mice (1, 7, 34). In these studies, CD4$^+$ CD25$^+$ T_{reg} cells were shown to control both CD4$^+$ Th1 and Th2 cellular immune responses. The role of CD4$^+$ CD25$^+$ T_{reg} cells during infection with *Leishmania* in humans has not been investigated directly; investigations have relied on the measurement of cytokines secreted by CD4$^+$ CD25$^+$ T_{reg} cells.

Most of our knowledge about cytokine interaction in humans has come from the studies on different clinical forms of leishmaniasis, and there is limited information about the development of cytokine responses in healthy individuals. In this regard, *Leishmania*-specific CD8$^+$ and CD4$^+$ T cells were isolated from PBMC of subjects who had never been previously exposed to *Leishmania* (9, 23), but the exact role of these cells in resistance versus susceptibility to infection is not yet estab-

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lished. Although IL-10- and IFN-γ-producing T cells, which recognize the *Leishmania* homologue of receptors of activated C kinases (LACK), have been detected in naive subjects (9, 23), only a limited amount of IL-10 was detected in response to live *L. guyanensis*, which might be due to the presence of a suppressive cytokine. In the present study, we demonstrated that TGF-β is produced by PBMC from healthy subjects in response to live *Leishmania* stimulation and that $CD4^+$ $CD25^+$ regulatory T cells are the TGF-β1-secreting cells.

MATERIALS AND METHODS

Subjects. Blood samples were obtained by venipuncture from 13 healthy controls and 15 patients suffering from leishmaniasis due to *L. guyanensis* (duration of lesion development, <3 months) and collected into sterile tubes (Veinoject; Teruven, Leuven, Belgium). All subjects were seronegative for human immunodeficiency virus. Absence of prior exposure to *Leishmania* was assessed based on (i) the absence of scars due to leishmaniasis upon clinical examination, (ii) no history of any stay in a country in which leishmaniasis is endemic, and (iii) no reactivity against soluble *Leishmania* antigen from *L. guyanensis* based on the inability of these healthy subjects to develop IFN-γ reactivity and specific antibodies against soluble *Leishmania* antigens (10).

Informed consent was obtained from the subjects, and the human guidelines of the "Comité Consultatif de Protection des Personnes dans la Recherche Médicale" (CCPRB) of Guadeloupe were followed (project 99-3).

Antigens. *Leishmania guyanensis* (M4147) and *Leishmania major* (MRHO/SU/59/P strain) promastigotes were cultured in biphasic rabbit blood agar (22). *L. major* LV 39 amastigotes were obtained as previously described (15). Extracellular proteins were removed from the parasite pellets by three washes with phosphate-buffered saline. Parasites were used at 10^6/ml. In some experiments, *L. major* promastigotes were rendered unable to replicate by a 5-min irradiation with UV radiation (UVC; λ, 253 nm; 200 mW s/cm^2) (27).

Reagents. The reagents for magnetic cell separation with anti-CD4-, anti-CD8-, and anti-mouse immunoglobulin G (IgG)-coated magnetic beads were obtained from Dynal (Compiègne, France). Mouse anti-human CD3 (UTCHT1; IgG1), CD25 (M-1251; IgG1), CD45RO (UCHL-1; IgG2a), DR (TÜ36; IgG2b), CCR4 (1C1; IgG1,), CD62L (Dreg56; IgG1), CCR7 (2H4; IgM), CD29, or β_1 integrin (HUTS-21; IgG2a), and CD49d, or α_4 integrin (9F10; IgG1) antibodies, and rat anti-human cutaneous lymphocyte antigen (CLA) (HECA-452; IgM) and anti-human β_7 integrin (FIB504; IgG2a) antibodies, were obtained from Pharmingen (San Diego, CA).

Neutralizing mouse anti-human TGF-β1 (TB21; IgG1) and anti-IL-10 (JES3-9D7; IgG1) monoclonal antibodies (MAbs) were obtained from Biosource International (Carmarillo, CA) and Pharmingen, respectively. The purified mouse IgG1 isotype control (107.3, IgG1; anti-trinitrophenol) was obtained from Pharmingen.

Mouse anti-CD4 (RPA-T4; IgG1) and anti-CD8 (RPA-T8; IgG1) conjugated to phycoerythrin for flow cytometric experiments were obtained from Pharmingen.

For T-cell activation, the stimulatory anti-CD3 (UTCHT1; IgG1) antibody was used at 2.5 μg/ml.

Cell isolation and activation. PBMC were isolated after venipuncture on a Ficoll-Hypaque gradient ($d = 1.077$), and $CD3^+$ and $CD3^-$ T cells were purified with a magnetic activated cell sorter from Dynal. Briefly, cells conjugated with an anti-CD3 MAb were suspended with anti-mouse IgG-coated magnetic microbeads and isolated after exposure to a magnetic field. The purity was 96%, as determined by fluorescence-activated cell sorter (FACS) analysis. $CD4^+$ and $CD8^+$ cells were purified and depleted from PBMC with CD4 and CD8 magnetic beads, as described by the manufacturer (Dynal). This resulted in 92% pure $CD8^+$ T cells and 95% pure $CD4^+$ T cells, as determined by FACS analysis. CD8 and CD4 MAbs were released with Detachabeads as described by the manufacturer (Dynal). The purified $CD8^+$ and $CD4^+$ T cells were then incubated with various mouse anti-human MAbs and isolated with magnetic beads conjugated with anti-mouse IgG or IgM MAbs. As anti-human CLA and anti-human β_7 integrin were produced as an IgM and IgG from rat, respectively, an intermediate step with mouse anti-rat IgM or an anti-rat IgG was added before separation. For the $CD4^+$ $CD25^+$ T-cell positive selection, isolated $CD4^+$ T cells were incubated with anti-CD25 MAb and anti-mouse IgG magnetic beads. The purity in all cases was >92%.

T cells purified positively and negatively (10^4 cells) were stimulated in the presence of autologous PBMC treated with mitomycin C (10^6 cells) used as antigen-presenting cells with or without antigens in RPMI medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 1 mg/ml streptomycin (all from Sigma), and 5% heat-inactivated human AB serum. In some experiments mouse anti-TGF-β1, anti-IL-10, and the isotype control were added at 5 μg/ml. $CD4^+$ $CD25^+$ T cells or $CD4^+$ $CD25^-$ T cells (5×10^5 cells) in the presence of 10^6 mitomycin C-treated autologous PBMC were stimulated with either anti-CD3 or *L. guyanensis* to analyze the cytokine profile of these cells.

Suppression assay and cytokine analysis. Cocultures of $CD4^+$ $CD25^+$ T cells and $CD4^+$ $CD25^-$ T cells were activated with either anti-CD3 or *L. guyanensis* to analyze the suppressive activity of $CD4^+$ $CD25^+$ T cells on cytokine production. Briefly, a constant number of $CD4^+$ $CD25^-$ T cells (5×10^5 cells) was cultured with $CD4^+$ $CD25^+$ T cells (2×10^5), which gave optimal suppression in preliminary experiments in the presence of mitomycin C-treated PBMC (data not shown). The supernatants for all the experiments were removed after 3 or 7 days of culture with anti-CD3 and *L. guyanensis*, respectively, and stored at −20°C. We analyzed cytokine production by specific IL-4, IL-10, IL-13, IFN-γ, and TGF-β1 enzyme-linked immunosorbent assays (ELISA) (Pharmingen) with a sensitivity of 10 pg/ml (except for TGF-β1, for which the assay sensitivity was 62.5 pg/ml).

Statistical analysis. The data were subjected to statistical analysis by the nonparametric Kruskal-Wallis test.

RESULTS

PBMC from naive subjects produce large amounts of TGF-β1 in response to *Leishmania* promastigotes. We examined cytokine production by PBMC from 13 healthy subjects in the presence of live *L. guyanensis* promastigotes. PBMC from 15 patients suffering from leishmaniasis due to infection with *L. guyanenis*, the *Leishmania* species most frequently isolated in French Guyana, were also analyzed. Figure 1A clearly shows that TGF-β1 is produced only by PBMC from healthy subjects stimulated with *L. guyanensis*. Production of TGF-β1 required live *L. guyanensis*, since PBMC stimulated with UV-irradiated *L. guyanensis* or with frozen and thawed *L. guyanensis* promastigotes did not induce TGF-β1 in PBMC from controls or localized cutaneous leishmaniasis (LCL) patients (data not shown). Furthermore, stimulation of PBMC with a 10-fold increase in UV-irradiated *L. guyanensis* (10^7) was not able to induce TGF–β1 production in PBMC (data not shown).

To determine whether TGF-β1 production by PBMC from healthy subjects is limited to stimulation with leishmaniae from the New World, we analyzed TGF-β1 production after stimulation with live promastigotes of *L. major*, a representative parasite from the Old World. Figure 1B shows that TGF-β1 is also produced by PBMC from healthy subjects stimulated with live *L. major* promastigotes.

Furthermore, the developmental stages—promastigotes versus amastigotes—involved in TGF-β1 production by PBMC from healthy subjects were analyzed. Since we are unable to obtain amastigotes from *L. guyanensis*, however, we compared TGF-β1 production after stimulation with either promastigotes or amastigotes from *L. major*. Interestingly, the levels of TGF-β1 produced in response to *L. major* amastigotes were lower than those obtained with the promastigote forms (Fig. 1B).

As previously described (9, 11), live *L. guyanensis* induced IFN-γ in PBMC in either controls or LCL patients, but no IL-4 was detectable in response to live *L. guyanensis* (data not shown). However, some IL-2 (18 ± 7 pg/ml) and IL-10 (35 ± 12 pg/ml) are detected in supernatants of PBMC from LCL patients stimulated with live *L. guyanensis*.

$CD4^+$ $C25^+$ T cells are the cells producing TGF-β1 in response to live *L. guyanensis*. To determine which cells pro-

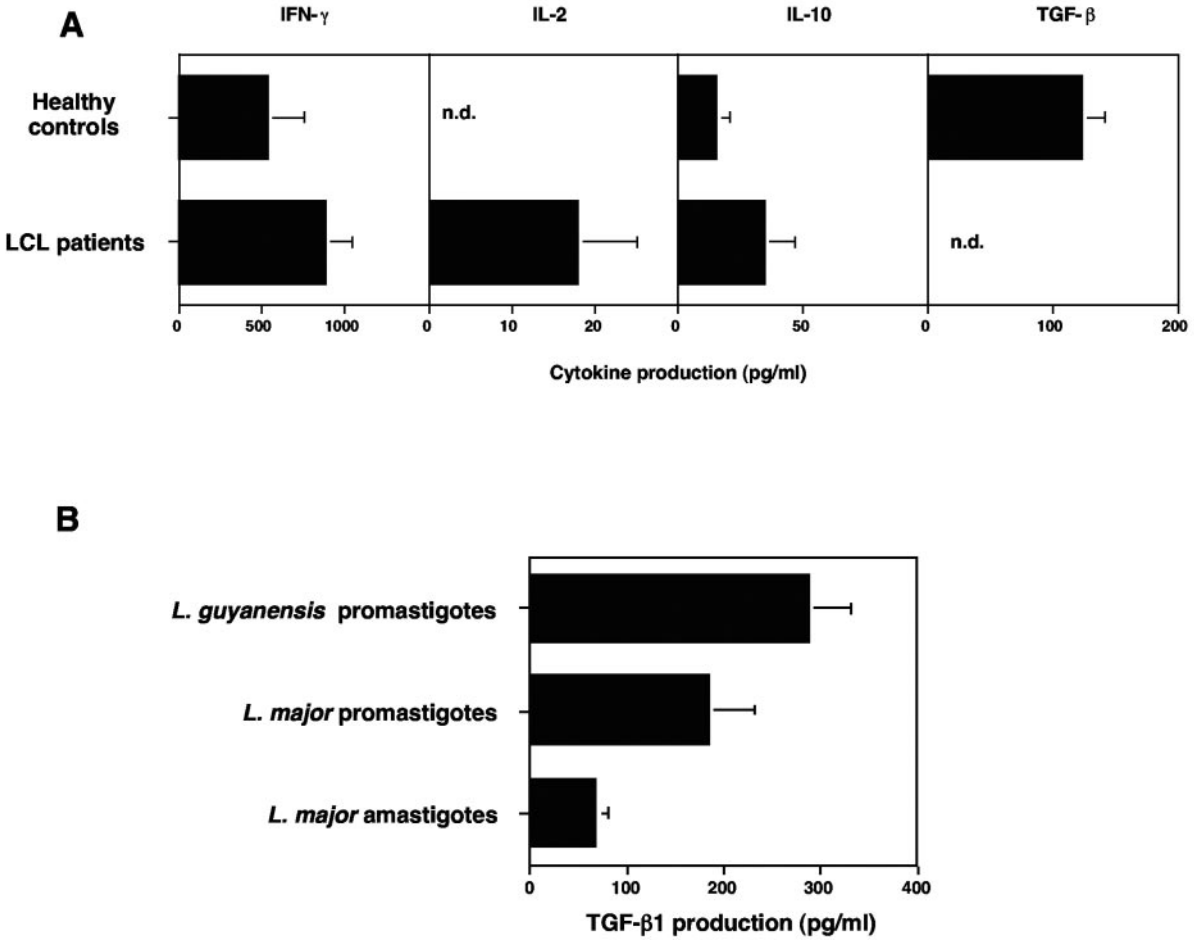


FIG. 1. Production of cytokines by PBMC in response to live *Leishmania* promastigotes. (A) Cytokines were produced by culture of PBMC (10^6/ml) from healthy controls and LCL patients in the presence of live *Leishmania guyanensis* promastigotes (10^6/ml) as described in Materials and Methods. Supernatants were harvested after 2 days for IL-2 and after 7 days for IFN-γ, IL-10, and TGF-β1 production. Cytokine production was analyzed by using specific ELISA. These ELISA had sensitivities of 10 pg/ml except for TGF-β1 (62.5 pg/ml). n.d., not detected. Production of cytokines by unstimulated cells was under the limit of detection for healthy controls. Unstimulated cells from LCL patients produced low levels of IFN-γ (54 ± 12 pg/ml) and IL-10 (12 ± 8 pg/ml), but IL-2 and TGF-β1 production was undetectable. (B) TGF-β1 production was analyzed by culture of PBMC from healthy controls in the presence of either live *L. guyanensis* or live *L. major* promastigotes or amastigotes (10^6/ml) as described above.

duced TGF-β1 in response to live *L. guyanensis* in PBMC from healthy subjects stimulated with live *L. guyanensis*, we analyzed cytokine production in the supernatants of unfractionated cells and of cells purified for CD3, CD4, and CD8. TGF-β1-producing cells were found to be $CD3^+$ $CD4^+$ T cells (Fig. 2A). Since a subset of TGF-β-producing $CD4^+$ T cells has been characterized by expression of the IL-2 receptor α chain (CD25), we analyzed TGF-β1 production by $CD25^+$ and $CD25^-$ $CD4^+$ T cells in response to live *L. guyanensis*. As shown in Fig. 2B, the TGF-β1-producing cells are $CD25^+$. Further characterization of the TGF-β-producing cells demonstrated that the TGF-β1 producers were found among the $CD45RO^+$ DR^+ $CD4^+$ T cells (Fig. 2B), which are phenotypically identical to the previously described $CD4^+$ T_{reg} cell population (30).

Recently, two distinct subsets of human T_{reg} cells have been identified in peripheral blood of healthy subjects based on the expression of α/β integrins: the $\alpha_4\beta_7^+$ T_{reg} cells produced large amounts of IL-10, whereas $\alpha_4\beta_1^+$ T_{reg} cells produced TGF-β (31). Thus, we characterized the expression of these integrins on the T_{reg} cells producing TGF-β1 in response to *L. guyanensis*. Figure 2C clearly demonstrates that these cells expressed the $\alpha_4\beta_1$ integrin.

We then assessed the expression on TGF-β1-secreting $CD4^+$ T cells of CLA and CC chemokine receptor 4 (CCR4), which drive the migration of T cells to the skin (14, 17). In addition, expression of CCR7 and CD62L, both necessary for migration to lymph nodes (4, 12), was evaluated. Results presented in Fig. 2D indicate that the TGF-β1-producing $CD4^+$ T cells are CLA^+ and $CCR4^+$, implying that these cells are able to recirculate into the skin. TGF-β1-secreting cells did not express CD62L or CCR7 molecules, since all the TGF-β1 produced in response to live *L. guyanensis* was detected in the supernatants of $CD62L^-$ and $CCR7^-$ $CD4^+$ T cells.

$CD4^+$ $CD25^+$ T cells produced TGF-β1 but not IL-10 in response to *L. guyanensis*. Since $CD4^+$ $CD25^+$ T cells that produced TGF-β1 are phenotypically similar to the previously described T_{reg} cells, we analyzed the cytokine profile of these

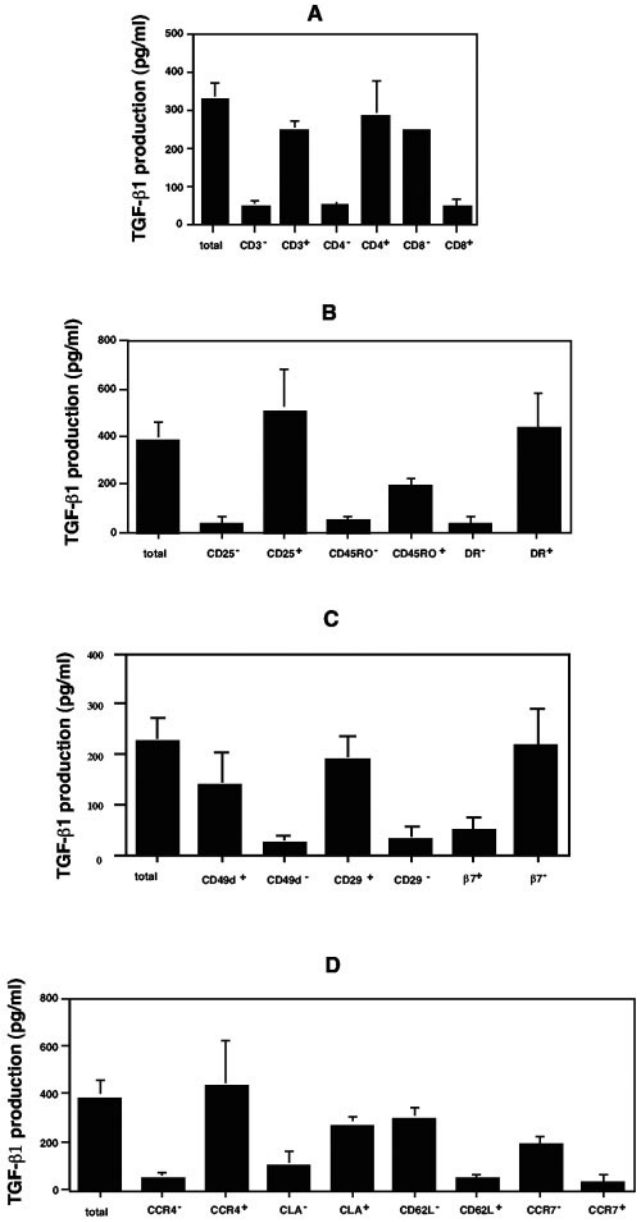


FIG. 2. Phenotype of the cells producing TGF-β1 in response to stimulation with live *L. guyanensis*. (A) TGF-β1 production was analyzed in supernatants of live *L. guyanensis*-stimulated unfractionated cells and in cells negatively and positively selected for CD3, CD4, and CD8 before antigen exposure by using magnetic beads as described in Materials and Methods (Dynal) This resulted in 96% pure $CD3^+$ T cells, 92% pure $CD8^+$ T cells, and 95% pure $CD4^+$ T cells, as determined by FACS analysis. (B, C, and D) $CD4^+$ T cells were purified from PBMC by using anti-CD4 magnetic beads (Dynal). This yielded 92% pure $CD4^+$ T cells. After release of CD4 MAbs with Detachabeads (Dynal), $CD4^+$ T cells were incubated with different mouse anti-human MAbs (Pharmingen). $CD4^+$ T cells positively and negatively purified for CD25, CD45RO, and DR (B), CD29 (β1 integrin), CD49d (α4 integrin), and β7 integrin (C), and CCR4, CLA, CD62L, and CCR7 (D) were stimulated with live *L. guyanensis*, and after 7 days, the supernatants were harvested for TGF-β1 determination as described in Materials and Methods. Data are means ± standard errors of the means for six healthy controls.

cells in comparison to that of $CD4^+$ $CD25^-$ T cells. As shown in Fig. 3, $CD4^+$ $CD25^+$ T cells produced IL-10 and TGF-β1 but not IL-2 and IFN-γ in response to anti-CD3 stimulation, corresponding to the cytokine profile described for human T_{reg} cells. Interestingly, the fact that $CD4^+$ $CD25^+$ T cells produced TGF-β1 but not IL-10 in response to *L. guyanensis* clearly reinforces results showing that this parasite stimulates a distinct subpopulation of $CD4^+$ $CD25^+$ T_{reg} cells expressing the $\alpha_4\beta_1$ integrin (Fig. 1).

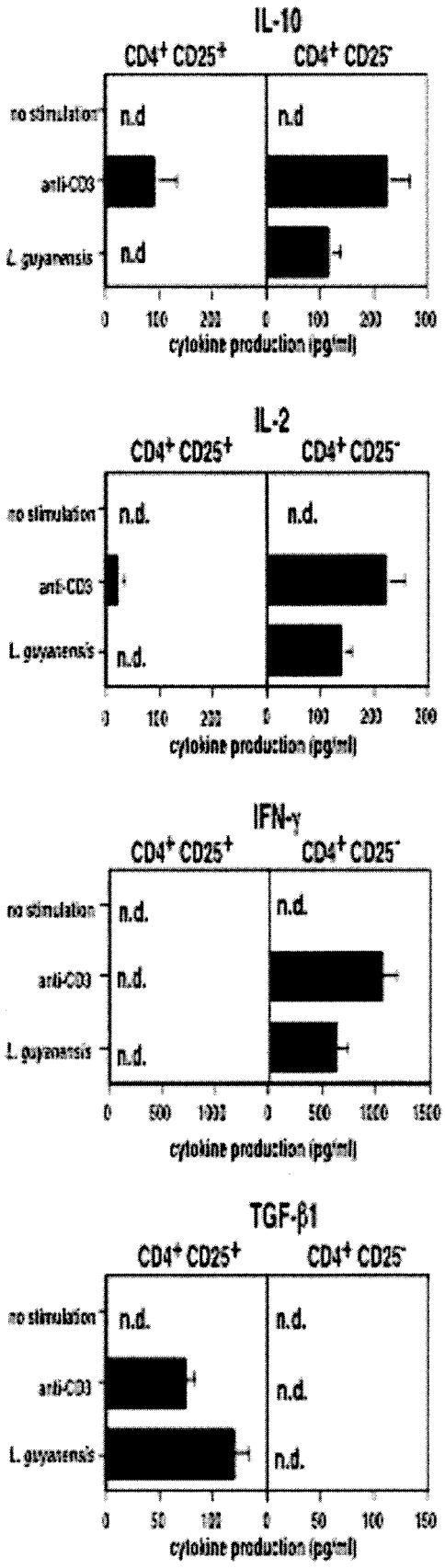
As expected, $CD4^+$ $CD25^-$ T cells produced IL-2, IL-10, and IFN-γ but not TGF-β1 in response to anti-CD3 stimulation (Fig. 3). If IFN-γ production by $CD4^+$ T cells in response to *L. guyanensis* was not surprising in subjects never exposed to *Leishmania*, since we and others have already demonstrated such cytokine production (9, 23), $CD4^+$ $CD25^-$ T cells produced IL-2 and IL-10 but not TGF-β1 in response to *L. guyanensis* stimulation. Since IL-2 and IL-10 were either undetectable or produced at very low levels in total PBMC stimulated with *L. guyanensis* (Fig. 1), these results suggest that IL-2 and IL-10 production in response to the parasite may be down regulated by the $CD4^+$ $CD25^+$ T cells.

$CD4^+$ $CD25^+$ T cells suppress IL-2 and IL-10 production by $CD4^+$ $CD25^-$ T cells through TGF-β1 production. To test the suppressive activity of $CD4^+$ $CD25^+$ T cells, we performed classical in vitro suppression assays by coculture of $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells in the presence of anti-CD3 or *L. guyanensis*. First, levels of cytokines were under the limit of detection in mitomycin C-treated APCs stimulated with *L. guyanensis* or anti-CD3. As shown in Fig. 4, $CD4^+$ $CD25^+$ T cells are able to inhibit the secretion of IL-2 and IL-10 by $CD4^+$ $CD25^-$ T cells in the presence of either anti-CD3 or *L. guyanensis*. However, $CD4^+$ $CD25^+$ T cells were able to suppress IFN-γ production by $CD4^+$ $CD25^-$ T cells in response to anti-CD3 (Fig. 4B) but were unable to inhibit the IFN-γ production of $CD4^+$ $CD25^-$ T cells in response to *L. guyanensis* (Fig. 4A).

Since the role of cytokines in mediating the suppressive effect of $CD4^+$ $CD25^+$ T cells is not clear, we performed coculture assays in the presence of anti-TGF-β1 and anti-IL-10 MAbs to analyze the mechanisms of suppression. Addition of anti-TGF-β1, anti-IL-10, or isotype control MAbs did not reverse suppression induced by $CD4^+$ $CD25^+$ T cells in the presence of anti-CD3 (Fig. 4B). However, addition of anti-TGF-β1, but not anti-IL-10 or isotype control MAbs, reversed the suppressive activity of $CD4^+$ $CD25^+$ T cells activated with *L. guyanensis* (Fig. 4A).

DISCUSSION

In the present study, we first demonstrated TGF-β1 production by PBMC from healthy subjects in response to live *L. guyanensis* and further demonstrated that the TGF-β1-producing cells are phenotypically and functionally identical to the $CD4^+$ $CD25^+$ T regulatory cells. TGF-β is recognized as a central immunoregulator used by the parasite to escape protective mechanisms in human and murine infection with *Leishmania*. Indeed, previous reports demonstrated a central role of endogenous TGF-β in the susceptibility of mice to *L. major*. TGF-β also increased the susceptibility of BALB/c mice to infection with *L. amazonensis* (6) and reverted the resistance of



C57BL/6 mice to *L. chagasi* infection (33). Furthermore, administration of anti-TGF-β MAbs blocked the development of lesions in mice susceptible to infection with *L. major* (21). In human infections, TGF-β has been detected in either active or chronic human cutaneous leishmaniasis (25), and macrophages from LCL patients were shown to produce TGF-β after infection with different *Leishmania* species (5). Since the amount of TGF-β1 produced by macrophages upon infection with *Leishmania* correlates with both the strain virulence and the multiplication of parasites within the macrophages, TGF-β production was associated with a virulence mechanism during infection with *Leishmania* (5). Little is known about TGF-β production in response to stimulation with *Leishmania* in healthy subjects. Our present results demonstrated that TGF-β1 is also produced in PBMC of healthy subjects.

Among the populations of cells which are able to produce TGF-β-1, the recently described T_{reg} cells have been analyzed extensively, mainly in the context of tolerance or autoimmunity (30). Several subsets of T_{reg} cells which express CD25 have been described (24). However, since no specific surface antigens have been identified, characterization of T_{reg} cells was done based on the pattern of secreted cytokines and the mechanism of suppression in vitro, although the transcription factor Foxp3 was reported to be a specific marker for $CD4^+$ $CD25^+$ T cells (13, 16, 18). Results presented in this paper clearly demonstrate that TGF-β1-producing cells generated in response to *L. guyanensis* are phenotipically and functionally similar to the human $CD4^+$ $CD25^+$ T_{reg} cells, since (i) they express CD25, CD45RO, and DR surface markers and (ii) they exert suppressive activity on $CD4^+$ $CD25^-$ T cells in vitro. We further show that $CD4^+$ $CD25^+$ T cells stimulated by *L. guyanensis* produced TGF-β1 but not IL-10. $CD4^+$ $CD25^+$ T_{reg} cells stimulated with anti-CD3, however, produced both TGF and IL-10, suggesting that the former population of $CD4^+$ $CD25^+$ T cells might be a particular subpopulation of T_{reg} cells.

The exact mechanisms by which $CD4^+$ $CD25^+$ T cells exert their suppressive activity are still controversial. Indeed, soluble suppressor cytokines such as IL-10 and TGF-β have been reported to be involved in this process in vivo, but cell contact between suppressor cells and the responder cells has been shown to be required in vitro (3, 8). Our results show that addition of neutralizing MAbs to TGF-β1 can reverse suppression of IL-2 and IL-10 production in response to *L. guyanensis* but not to anti-CD3 stimulation of $CD4^+$ $CD25^+$ T cells, demonstrating that TGF-β1 is involved in the suppressive activity of *L. guyanensis*-stimulated $CD4^+$ $CD25^+$ T cells in vitro (30). These results reinforce the hypothesis that *L. guyanensis* could activate a subpopulation of T_{reg} cells. In this context,

FIG. 3. Cytokine secretion profile of $CD25^+$ and $CD25^-$ $CD4^+$ T cells. $CD4^+$ T cells were purified from PBMC using anti-CD4 magnetic beads (Dynal). This yielded 94% pure $CD4^+$ T cells. After release of CD4 MAbs with Detachabeads (Dynal), $CD4^+$ T cells were incubated with an anti-CD25 MAb (Pharmingen) and purified on magnetic beads with anti-mouse IgG antibodies. $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells (5×10^5) were stimulated with either anti-CD3 MAbs or live *L. guyanensis*. Supernatants were collected after 3 and 7 days of culture with anti-CD3 and live *L. guyanensis*, respectively. Data are means ± standard errors of the means for six healthy controls. n.d., not detected.

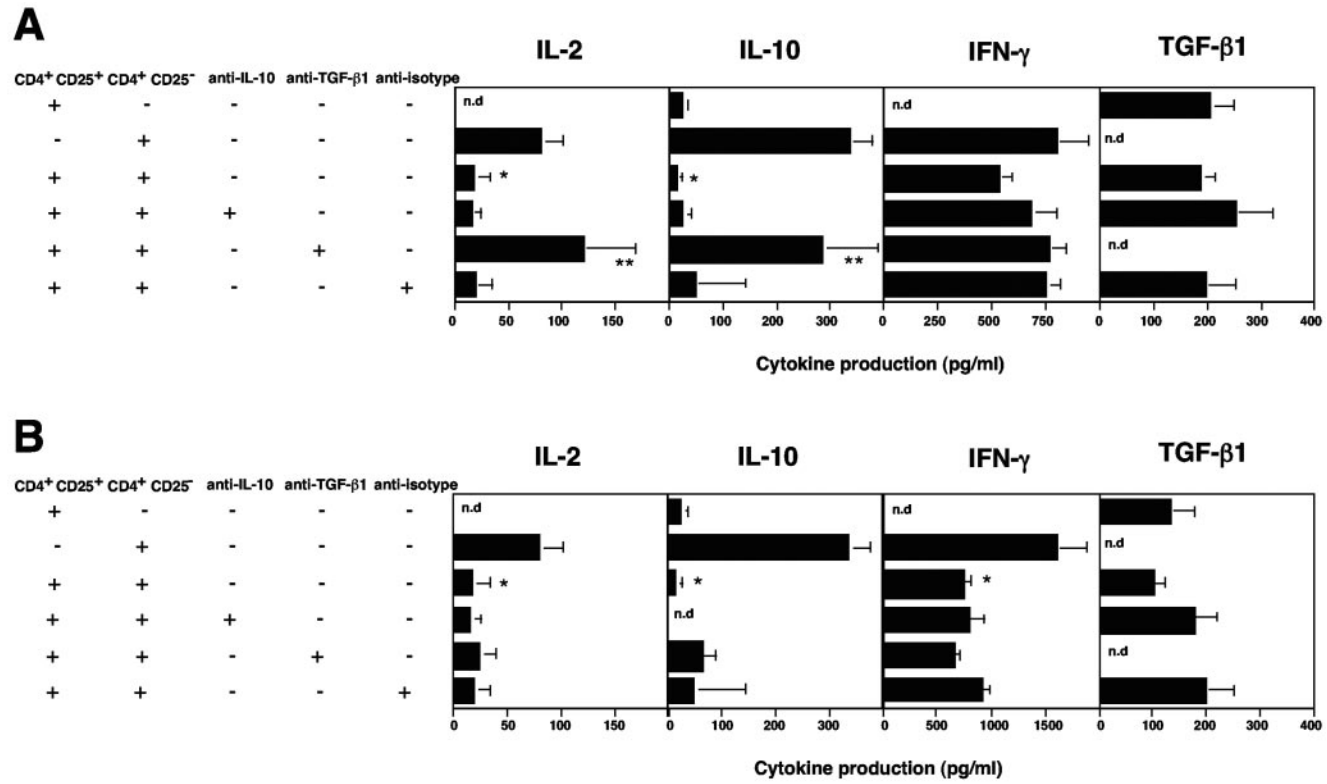


FIG. 4. Suppression of cytokine production in $CD4^+$ $CD25^-$ T-cell responses by $CD4^+$ $CD25^+$ T cells. $CD4^+$ T cells were purified from PBMC using anti-CD4 magnetic beads (Dynal). This yielded 95% pure $CD4^+$ T cells. After release of CD4 MAbs with Detachabeads (Dynal), $CD4^+$ T cells were incubated with an anti-CD25 MAb (Pharmingen) and purified on magnetic beads with anti-mouse IgG antibodies. Purified $CD4^+$ $CD25^-$ T cells were then activated with either live *L. guyanensis* (A) or an anti-CD3 MAb (B) in the presence or absence of $CD4^+$ $CD25^+$ T cells as described in Materials and Methods. After 3 and 7 days of culture, supernatants were collected for analysis of cytokine production. Some cultures were carried out in the presence of either anti-IL-10, anti-TGF-β1, or an isotype control MAb (5 μg/ml) to analyze the role of these cytokines in the suppressor activity of $CD4^+$ $CD25^+$ T cells. n.d., not detected. *, statistical significance for cocultures of $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells versus cultures of $CD4^+$ $CD25^-$ T cells alone ($P < 0.05$). **, statistical significance for cultures of $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells containing neutralizing antibodies versus cultures without antibodies ($P < 0.05$).

$CD4^+$ $C25^+$ T-cell clones producing high levels of TGF-β1 have been identified recently in humans, and their suppressive activity was reported to be partially dependent on TGF-β (20). Thus, *L. guyanensis* could be able to stimulate such a subpopulation of $CD4^+$ $CD25^+$ T cells. This result is reinforced by the fact that TGF-β1-producing cells expressed $\alpha_4\beta_1$ integrin in response to live *L. guyanensis*. The $\alpha_4\beta_1$ integrin has been recently described on a subpopulation of human T_{reg} cells (31). Since $\alpha_4\beta_1$ integrin binds to vascular cell adhesion molecule-1 (CD106; VCAM-1), which is induced on the endothelium of inflamed tissues, we could hypothesize that the TGF-β1-producing cells migrate to the inflamed tissues to inhibit the T-cell response to parasites.

The $CD4^+$ $C25^+$ T cells generated in response to *L. guyanensis* have been identified in PBMC, and they were shown to express CLA and CCR4; therefore, they should be able to migrate into the inflamed skin where *Leishmania* parasites exert their functions. The lack of detection of TGF-β1 production in supernatants of *L. guyanensis*-stimulated PBMC from infected patients might be explained by a specific recruitment of this subpopulation of $CD4^+$ $CD25^+$ T cells into the lesions. Analysis of lesions might give a response; however, the finding that murine $CD4^+$ $C25^+$ T cells preferentially accumulate in the skin during the chronic phases of infection with *L. major* is in accordance with this hypothesis (7). We cannot rule out the possibility that the suppressor function of human $CD4^+$ $CD25^+$ regulatory T cells in peripheral blood of patients with leishmaniasis is defective, as was recently reported for patients suffering from autoimmune diseases (19, 32).

In conclusion, TGF-β1 production by a subpopulation of $CD4^+$ $C25^+$ T cells showing a regulatory phenotype was identified in PBMC from healthy subjects stimulated with *Leishmania*, but the role of these cells in infection and disease is not yet fully understood. The immune responses detected in healthy subjects could, however, reflect the propensity of *Leishmania* to infect (either clinically or subclinically) most exposed individuals. The fact that TGF-β1 is known to downregulate a number of immune responses associated with protective mechanisms (e.g., macrophage activation) is in accordance with the second hypothesis.

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